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Triterpene saponins from *Eryngium kotschyi*

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ABSTRACT

Four new oleanane-type saponins 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucuronopyranosyl-22-*O*-β,β-dimethylacryloylA1-barrigenol (**1**), 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucuronopyranosyl-22-*O*-angeloylA1-barrigenol (**2**), 3-*O*-β-D-glucopyranosyl-(1 → 2)-[β-D-glucopyranosyl-(1 → 6)]-β-D-glucopyranosyl-21,22,28-*O*-triacetyl-(3β,21β,22α)-olean-12-en-16-one (**3**), and 3-*O*-β-D-glucopyranosyl-(1 → 2)-glucopyranosyl-22-*O*-β-D-glucopyranosylsteganogenin (**4**), along with the known 3-*O*-β-D-galactopyranosyl-(1 → 2)-[α-L-arabinopyranosyl-(1 → 3)]-β-D-glucuronopyranosyl-22-*O*-angeloylA1-barrigenol and 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucuronopyranosyloleanolic acid, were isolated from a methanol extract of the roots of *Eryngium kotschyi* by multiple chromatographic steps. Saponins **3** and **4** are unusual by the original structure of their aglycon. Compound **3** possessed an oleanane-type skeleton with a 21,22,28-triacetylation and a ketone function at the C-16 position. For compound **4**, the 17,22-seco-oleanolic acid skeleton is rarely found in natural saponins.

1. Introduction

Eryngium is an important genus of the Apiaceae family, subfamily Saniculoideae according to the APGIII classification (The Angiosperm phylogeny group, 2009), containing about 250 species, distributed in Eurasia, North Africa, North and South America, and Australia (Wörz, 2004). As an outcome of this wide distribution, they are well known plants in ethnobotanical culture in the world and also in Turkey. *Eryngium campestre*, *Eryngium maritimum* and related species, are known as «boğadikeni» in Turkish folk-medicine, and are used as antitussive, diuretic, appetizer, stimulant, aphrodisiac and against scorpion venom (Küpeli et al., 2006). Phytochemical studies performed on *Eryngium* species revealed a triterpene saponin richness, and various secondary metabolites (Kartal et al., 2005, 2006). Most of the saponins isolated from *Eryngium* species possess hydroxylated oleanane-type aglycons such as barringtogenol C, A1-barrigenol or R1-barrigenol (Hiller et al., 1975; Kartal et al., 2005, 2006). In previous biological studies, *Eryngium kotschyi* showed significant cytotoxic (Yurdakök and Baydan, 2013), antinociceptive, anti-inflammatory (Küpeli et al., 2006), antibacterial and antioxidant activities (Yurdakök and Baydan, 2013; Yurdakök et al., 2014). We thus described in this paper the isolation of six saponins from a methanolic root extract of *E. kotschyi* Boiss. by successive chromatographic methods. The structural determinations of four new (1–4) and two known triterpene saponins were performed by a detailed analysis of their spectral data including 600 MHz 2D NMR experiments (COSY, TOCSY, NOESY, HSQC, HMBC), and mass spectrometry. Chemotaxonomic significance will be discussed.

2. Results and discussion

A methanolic root extract of *E. kotschyi* Boiss. was subjected to multiple chromatographic steps over silica gel yielding four new compounds **1**–**4** (Fig. 1) and the known 3-*O*-β-D-galactopyranosyl-(1 → 2)-[α-L-arabinopyranosyl-(1 → 3)]-β-D-glucuronopyranosyl-22-*O*-angeloylA1-barrigenol (Bäcker et al., 2013) and 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucuronopyranosyloleanolic acid (Melek et al., 2003). The elucidation of their structures was performed mainly by 600 MHz 2D NMR analysis (^{1}H–^{1}H COSY, TOCSY, NOESY, HSQC, HMBC), in combination with mass spectrometry (HRESIMS and FABMS). All saponins were isolated as white amorphous powders.

The HRESIMS of compound **1** established its molecular formula as $C_{47}H_{74}O_{16}$. Its FABMS (negative-ion mode) showed a quasimolecular ion peak $[M-H]^-$ at *m/z* 893 indicating a molecular weight

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of 894 and another fragment ion peak at m/z 747 [(M−H) − 146]$^-$, corresponding to the elimination of one 6-desoxyhexosyl moiety.

The ^{1}H NMR spectrum of the aglycon part of **1** showed signals for seven angular methyl groups as singlets at δ_H 0.73, 0.88, 0.98, 0.98, 1.14, 1.18, and 1.78, one olefinic proton at δ_H 5.49 (*br t*, J = 3.0 Hz) (H-12), four oxygen-bearing methine protons at δ_H 3.19 (*dd*, J = 12.3, 4.2 Hz) (H-3), 4.21 (*d*, J = 3.7 Hz) (H-15), 4.46 (*d*, J = 3.7 Hz) (H-16), and 6.05 (*dd*, J = 12.0, 5.7 Hz) (H-22), and one primary alcoholic function at δ_H 3.55 (*d*, J = 10.5 Hz), 3.70 (*d*, J = 10.5 Hz) (H_2-28). A HMBC correlation between δ_H 4.21 (H-15) and δ_C 143.9 (C-13) and a COSY correlation between δ_H 4.21 (H-15) and 4.46 (H-16), allowed the location of two secondary alcoholic functions at C-15 and C-16. Another HMBC cross-peak between δ_H 6.05 (*dd*, J = 12.0, 5.7 Hz) (H-22) and δ_C 74.5 (C-16) confirmed the location of the third secondary alcoholic function at C-22. A deshielded signal at δ_H 3.55 (*d*, J = 10.5 Hz), 3.70 (*d*, J = 10.5 Hz) (H_2-28) in comparison with a free methyl group, suggested a primary alcoholic function at the C-28 position. The configurations of C-3, C-15, C-16, and C-22 of the aglycon was determined by the connectivities observed in the NOESY spectrum between H-3α and H_3-23α, between H-15β and H_3-26β, between H-16β and H_3-26β, and between H-22β and H_3-30β. Moreover, the multiplicity as a doublet (J = 3.7 Hz) of the H-15 at δ_H 4.21 and H-16 at δ_H 4.46 suggested an axial/equatorial coupling, with an β-axial orientation of the H-15 and a β-equatorial orientation of the H-16.

The structure of the aglycon of **1** was thus recognized to be the triterpene A1-barrigenol and was in full agreement with literature data (Table 1) (Bäcker et al., 2013).

The deshielded chemical shift of H-22 at δ_H 6.05 (*dd*, J = 12.0, 5.7 Hz) and a HMBC cross-peak at δ_H 6.05 (*dd*, J = 12.0, 5.7 Hz, H-22)/δ_C 167.0 suggested that the C-22 was acylated. The substituent was composed by two vinylic methyl groups at δ_H 1.60 (*s*) and 2.11 (*s*), which correlated in the HMBC spectrum with one ethylenic quaternary carbon at δ_C 155.7 and an ethylenic methine carbon at δ_C 117.0. These data revealed the presence of a *β,β*-dimethylacryloyl group acylating the C-22 position (Table 1) (Manase et al., 2013).

The monosaccharides obtained by acid hydrolysis of **1** were identified by comparison on TLC with authentic samples as glucuronic acid (GlcA) and rhamnose (Rha). The absolute configurations of the sugars were determined to be D for GlcA and L for Rha by GC analysis by a method previously described (Hara et al., 1987). The relatively large $^3J_{H\text{-}1,H\text{-}2}$ value of the GlcA (7.2 Hz) in its pyranose form indicated a β anomeric orientation. The large $^1J_{H\text{-}1,C\text{-}1}$ value of the Rha (166 Hz) confirmed that the anomeric proton was equatorial (α-pyranoid anomeric form).

The ^{1}H NMR spectrum of **1** in the sugar region displayed signals of two anomeric protons at δ_H 4.76 (*d*, J = 7.2 Hz) and 5.87 (*br s*), which gave correlations in the HSQC spectrum with two anomeric carbon signals at δ_C 106.3 and 101.5, respectively. The ring protons of the monosaccharide residues were assigned starting from the readily identifiable anomeric protons by means of the ^{1}H-^{1}H COSY, TOCSY, HSQC, HMBC experiments. Units of one β-D-glucuronopyranosyl and one α-L-rhamnopyranosyl were thus identified (Table 2). A HMBC correlation at δ_H 4.76 (*d*, J = 7.2 Hz, GlcA-1)/δ_C 88.9 (C-3) and a NOESY cross-peak at δ_H 4.76 (*d*, J = 7.2 Hz, GlcA-1)/δ_H 3.19 (*dd*, J = 12.3, 4.2, H-3), revealed an *O*-heterosidic linkage between the C-3 position of the aglycon and a glucuronopyranosyl moiety. Another correlation in the HMBC spectrum between δ_H 5.87 (*br s*, Rha-1) and δ_C 80.0 (GlcA-4), and the reverse one between δ_H 4.51 (*t*, J = 8.1 Hz) (GlcA-4) and δ_C 101.5 (Rha-1), proved the (1 → 4) linkage between Rha and GlcA.

On the basis of the above results, the structure of **1** was elucidated as the new 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucuronopyranosyl-22-*O*-β,β-dimethylacryloylA1-barrigenol.

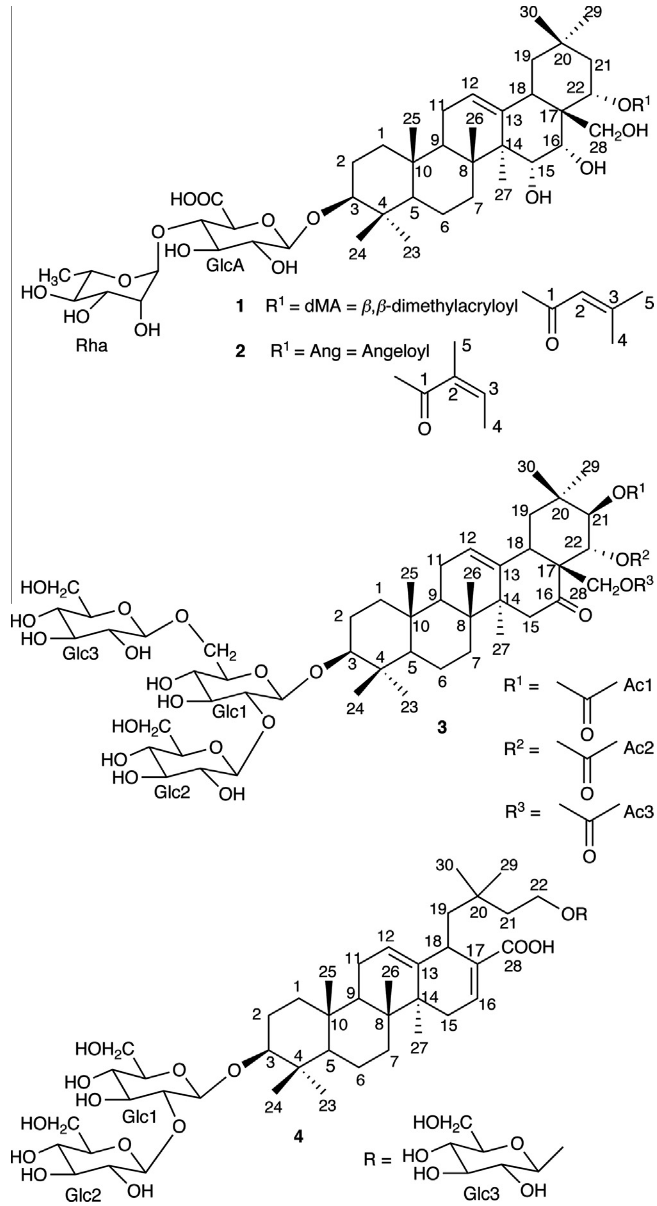

Fig. 1. Structures of compounds **1**–**4**.

The HRESIMS of compound **2** established its molecular formula as $C_{47}H_{74}O_{16}$, as compound **1**. Its FABMS (negative-ion mode) showed the same quasimolecular ion peak [M−H]$^-$ as **1** at m/z 893 indicating a molecular weight of 894. All the NMR signals observed for **2** are comparable with those of **1** excepted the residue acylating the C-22 position (Tables 1 and 2). In the COSY ^{1}H-^{1}H spectrum, two vinylic methyl groups at δ_H 1.82 (*br s*) and δ_H 1.98 (*br dd*, J = 7.1, 1.0 Hz) correlated with one ethylenic methine at δ_H 5.81 (*qd*, J = 7.1, 1.0 Hz), revealing the presence of an angeloyl group (Voutquenne et al., 1998). These observations were used to assign the structure of **2** as 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucuronopyranosyl-22-*O*-angeloylA1-barrigenol, a new natural compound.

The HRESIMS of compound **3** established its molecular formula as $C_{54}H_{84}O_{23}$. Its FABMS (negative-ion mode) showed a quasimolecular ion peak [M−H]$^-$ at m/z 1099 indicating a molecular weight of 1100. It displayed other fragment ion peaks at m/z 1056 [(M−H) − 43]$^-$ and 894 [(M−H) − 43 − 162]$^-$, which corresponded to the successive elimination of one acetyl group and one hexosyl moiety.

Table 1
^{13}C, ^{1}H NMR spectroscopic data of the aglycon moieties and acylating groups of **1**–**4** in pyridine-d_5 (δ_H ppm, *J* in Hz).[a]

	1			2			3		4	
	δ_C	δ_H		δ_C	δ_H		δ_C	δ_H	δ_C	δ_H
1	38.6	0.69, 1.28		38.6	0.69, 1.28		38.4	0.91, 1.47 *br d* (12.9)	38.2	0.69, 1.26
2	26.1	1.69, 2.07		26.1	1.69, 2.07		26.2	1.85, 2.27 *m*	26.1	1.75, 2.12 *m*
3	88.9	3.19 *dd* (12.3, 4.2)		88.8	3.19 *dd* (12.3, 4.2)		89.1	3.21 *dd* (11.6, 4.0)	89.4	3.19 *dd* (11.7, 4.0)
4	39.0	–		39.0	–		39.2	–	39.2	–
5	55.1	0.72		55.1	0.72		55.2	0.59 *br d* (11.7)	55.7	0.60 *br d* (11.7)
6	18.4	1.41, nd		17.2	1.41, nd		17.9	1.16, 1.36	18.2	1.19, 1.40
7	36.2	1.89, 1.99		36.2	1.89, 1.99		32.1	1.02, 1.25	32.5	1.21, 1.26
8	41.1	–		41.1	–		40.0	–	39.5	–
9	46.8	1.60		46.8	1.60		46.2	1.42	47.1	1.38
10	36.6	–		36.6	–		36.3	–	36.7	–
11	23.7	1.75, 1.82		23.7	1.75, 1.82		23.5	1.76, 1.85	23.1	1.52, 1.74
12	124.7	5.49 *br t* (3.0)		124.7	5.48 *br t* (3.0)		126.5	5.42 *br t* (3.1)	121.3	5.42 *br t* (2.9)
13	143.9	–		143.9	–		138.0	–	142.9	–
14	47.4	–		47.4	–		48.7	–	43.5	–
15	67.2	4.21 *d* (3.7)		67.2	4.19 *d* (3.6)		44.4	1.88 *d* (13.3), 2.79 *d* (13.3)	32.3	1.82 *d* (18.1), 2.36 *dd* (18.1, 6.7)
16	74.5	4.46 *d* (3.7)		74.1	4.45 *d* (3.6)		209.2	–	138.8	7.18 *dd* (6.7, 3.0)
17	44.8	–		44.8	–		56.8	–	143.0	–
18	41.4	2.95 *dd* (12.6, 3.0)		41.4	2.95 *dd* (12.5, 3.0)		47.2	3.04 *dd* (13.8, 3.1)	42.3	3.87
19	46.8	1.26, 2.79 *t* (12.6)		45.3	1.80, 2.66 *t* (12.5)		45.2	1.32, 1.90	45.1	1.46 *dd* (13.8, 4.6), 1.63 *dd* (13.8, 7.7)
20	31.6	–		31.6	–		35.9	–	33.3	–
21	41.3	1.94, 2.68 *t* (12.0)		41.1	1.91, 2.64 *t* (11.4)		75.8	5.99 *d* (10.5)	41.4	1.83, 1.93 *m*
22	72.4	6.05 *dd* (12.0, 5.7)		71.4	6.00 *dd* (11.4, 6.0)		73.1	5.54 *d* (10.5)	66.6	3.77 *m*, 4.34 *m*
23	27.8	1.14 *s*		27.8	1.14 *s*		27.7	1.13 *s*	27.7	1.14 *s*
24	16.7	0.88 *s*		16.7	0.88 *s*		16.4	1.02 *s*	16.3	0.97 *s*
25	15.4	0.73 *s*		15.4	0.73 *s*		15.2	0.75 *s*	15.2	0.63 *s*
26	17.2	0.98 *s*		17.2	0.98 *s*		16.6	0.91 *s*	17.1	0.44 *s*
27	21.0	1.78 *s*		20.9	1.75 *s*		26.6	1.21 *s*	28.1	1.23 *s*
28	62.6	3.55 *d* (10.5), 3.70 *d* (10.5)		62.5	3.50 *d* (11.7), 3.69 *d* (11.7),		67.8	4.52 *d* (11.6), 4.75 *d* (11.6)	170.0	–
29	33.1	0.98 *s*		33.1	0.94 *s*		28.8	0.89 *s*	28.0	1.01 *s*
30	24.8	1.18 *s*		24.8	1.08 *s*		18.9	1.07 *s*	28.3	0.93 *s*
dMA-1	167.0	–	Ang-1	168.0	–	Ac1-1	170.6	–		
2	117.0	5.54 *br s*	2	129.0	–	2	20.3	2.08 *s*		
3	155.7	–	3	136.5	5.81 *qd* (7.1, 1.0)	Ac2-1	170.6	–		
4	20.0	2.11 *s*	4	15.5	1.98 *br dd* (7.1, 1.0)	2	20.4	2.07 *s*		
5	26.7	1.60 *s*	5	20.6	1.82 *br s*	Ac3-1	171.2	–		
						2	20.7	2.03 *s*		

Nd: not determined.
[a] Overlapped proton signals are reported without designated multiplicity.

The ^{1}H NMR spectrum of the aglycon part of **3** showed signals for seven angular methyl groups as singlets at δ_H 0.75, 0.89, 0.91, 1.02, 1.07, 1.13 and 1.21, one olefinic proton at δ_H 5.42 (*br t*, *J* = 3.1 Hz) (H-12), three oxygen-bearing methine protons at δ_H 3.21 (*dd*, *J* = 11.6, 4.0 Hz) (H-3), 5.54 (*d*, *J* = 10.5 Hz) (H-22), and 5.99 (*d*, *J* = 10.5 Hz) (H-21), and one primary alcoholic function at δ_H 4.52 (*d*, *J* = 11.6 Hz), 4.75 (*d*, *J* = 11.6 Hz) (H_2-28). In the HMBC spectrum, the two methyl protons at δ_H 0.89 (*s*) (H_3-29) and 1.07 (*s*) (H_3-30) showed a correlation with a oxygen-bearing methine carbon at δ_C 75.8 that was assigned at position 21. A COSY correlation at δ_H 5.99 (*d*, *J* = 10.5 Hz) (H-21)/δ_H 5.54 (*d*, *J* = 10.5 Hz) (H-22), and a HMBC cross-peak at δ_H 4.52 (*d*, *J* = 11.6), 4.75 (*d*, *J* = 11.6) (H_2-28)/δ_C 47.2 (H-18), allowed the location of the second secondary alcoholic function at C-22 and the primary alcoholic function at C-28, respectively. The configurations of C-21 and C-22 was determined from the cross-peaks observed in the NOESY spectrum between H-21α and H_3-29α, and between H-22β and H_3-30β. In the ^{13}C NMR spectrum, a signal at δ_C 209.2 revealed a ketone function, located at the C-16 position according to the HMBC connectivities at δ_H 1.88 (*d*, *J* = 13.3 Hz), 2.79 (*d*, *J* = 13.3 Hz) (H_2-15)/δ_C 209.2 (H-16), and δ_H 4.52 (*d*, *J* = 11.6 Hz) (H-28)/δ_C 209.2 (H-16). The partial structure of the aglycon of **3** was thus identified as 3β,21β,22α,28-tetrahydroxy-olean-12-en-16-one (Table 1), a 16-oxydated derivative of the aglycon barringtogenol C (Pal et al., 1994). The 16-oxo function was already found in an aglycon isolated from another species of *Eryngium*, *Eryngium yuccifolium* (Wang et al., 2013; Zhang et al., 2008).

Signals of three acetyl groups remaining in the ^{1}H and ^{13}C NMR spectra, and the deshielded chemical shift of H-21 at δ_H 5.99, H-22 at δ_H 5.54, and H_2-28 at δ_H 4.52, 4.75, suggested acetylations at these positions. A HMBC cross-peak at δ_H 5.99 (H-21)/δ_C 170.6 and a NOESY cross-peak at δ_H 5.99 (H-21)/δ_H 2.08 (H_3-Ac1-2), and HMBC cross-peaks at δ_H 5.54 (H-22)/δ_C 170.6 and at δ_H 2.07 (H_3-Ac2-2)/δ_C 170.6, allowed the linkage between the acetyl groups Ac1 and Ac2 at C-21 and C-22 positions, respectively. The C-28 position is thus acylated by the last Ac3 moiety. The structure of the aglycon of **3** was elucidated as 21,22,28-*O*-triacetyl-(3β,21β,22α)-olean-12-en-16-one, described for the first time from a natural source.

The monosaccharides obtained by acid hydrolysis of **3** were identified by comparison on TLC with an authentic sample as glucose (Glc). Their absolute configuration was determined to be D by GC. The relatively large $^{3}J_{H\text{-}1,H\text{-}2}$ values of the Glc (7.1–7.9 Hz) in their pyranose form indicated a β anomeric orientation (Table 2).

The ^{1}H NMR spectrum of **3** displayed signals of three anomeric protons at δ_H 4.76 (*d*, *J* = 7.1 Hz), 4.96 (*d*, *J* = 7.9 Hz) and 5.28 (*d*, *J* = 7.6 Hz), which gave correlations in the HSQC spectrum with three anomeric carbon signals at δ_C 104.6, 104.7 and 104.8, respectively. Units of three β-D-glucopyranosyl moieties were identified (Table 2). A HMBC correlation at δ_H 4.76 (*d*, *J* = 7.1 Hz,

Table 2
^{13}C, ^{1}H NMR spectroscopic data of the sugar moieties of **1–4** in pyridine-d_5 (δ_H ppm, J in Hz).[a]

	1		2		3		4	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
3-*O*-GlcA-1	106.3	4.76 *d* (7.2)	106.3	4.77 *d* (7.3)				
2	75.2	4.03 *dd* (8.1, 7.2)	75.2	4.03 *dd* (8.2, 7.3)				
3	76.1	4.11 *t* (8.1)	76.1	4.11 *t* (8.2)				
4	80.0	4.51 *t* (8.1)	80.0	4.52 *t* (8.2)				
5	78.4	4.36 *d* (8.1)	78.3	4.36 *d* (8.2)				
6	175.9		175.8					
Rha-1	101.5	5.87 br s	101.5	5.87 br s				
2	71.6	4.86 br s	71.6	4.87 br s				
3	71.7	4.54 *dd* (8.7, 3.1)	71.7	4.54 *dd* (8.7, 3.1)				
4	73.2	4.15 *t* (8.7)	73.2	4.15 *t* (8.7)				
5	69.7	4.82 *dq* (8.7, 6.0)	69.8	4.82 *dq* (8.7, 6.1)				
6	18.2	1.51 *d* (6.0)	18.0	1.51 *d* (6.1)				
3-*O*-Glc1-1					104.6	4.76 *d* (7.1)	103.9	4.76 *d* (7.4)
2					81.6	4.15	81.5	4.17
3					77.6	4.21	77.4	4.22
4					70.7	3.98	70.8	3.97
5					76.1	4.00 *m*	77.8	3.82 *m*
6					69.7	4.20, 4.72 *br d* (11.0)	62.0	4.18, 4.40 *br d* (11.9)
Glc2-1					104.8	5.28 *d* (7.6)	104.5	5.27 *d* (7.4)
2					76.1	3.98	76.0	3.96
3					77.4	4.14	77.3	4.11 *t* (9.1)
4					71.3	4.09 *t* (9.2)	71.2	4.04 *t* (9.5)
5					77.8	3.84 *m*	77.6	3.81 *m*
6					60.9	4.19, 4.39 *dd* (12.1, 2.0)	62.2	4.21, 4.40 *br d* (11.9)
Glc3-1					104.7	4.96 *d* (7.9)	103.9	4.77 *d* (7.4)
2					74.5	3.94 *dd* (8.8, 7.9)	76.0	3.92 *dd* (8.6, 7.4)
3					77.4	4.13	77.3	4.08 *t* (8.6)
4					71.2	4.03 *t* (9.0)	71.2	4.00 *t* (8.6)
5					77.8	3.85 *m*	77.4	3.87 *m*
6					62.2	4.27 *dd* (11.4, 5.2), 4.43 *dd* (11.4, 1.9)	62.2	4.16, 4.40 *br d* (11.9)

[a] Overlapped proton signals are reported without designated multiplicity.

Glc1-1)/δ_C 89.1 (C-3) and a NOESY cross-peak at δ_H 4.76 (*d*, J = 7.1 Hz, Glc1-1)/δ_H 3.21 (*dd*, J = 11.6, 4.0 Hz, H-3), confirmed the linkage with the aglycon. Other correlations in the HMBC spectrum between δ_H 5.28 (*d*, J = 7.6 Hz, Glc2-1) and δ_C 81.6 (Glc1-2), and between δ_H 4.96 (*d*, J = 7.9 Hz, Glc3-1) and δ_C 69.7 (Glc1-6), showed the (1 → 2) and (1 → 6) linkages between the three β-D-glucopyranosyl moieties. Two NOESY cross-peaks at δ_H 5.28 (*d*, J = 7.6 Hz, Glc2-1)/δ_H 4.15 (Glc1-2), and at δ_H 4.96 (*d*, J = 7.9 Hz, Glc3-1) and δ_H 4.20, 4.72 (*br d*, J = 11.0 Hz, Glc1-6) proved these conclusions.

The structure of the new compound **3** was thus established as 3-*O*-β-D-glucopyranosyl-(1 → 2)-[β-D-glucopyranosyl-(1 → 6)]-β-D-glucopyranosyl-21,22,28-*O*-triacetyl-(3β,21β,22α)-olean-12-en-16-one.

The HRESIMS of compound **4** established its molecular formula as $C_{48}H_{78}O_{19}$. Its FABMS (negative-ion mode) displayed a quasimolecular ion peak $[M-H]^-$ at *m/z* 957 indicating a molecular weight of 958, and other fragment ion peaks at *m/z* 795 $[(M-H) - 162]^-$ and 633 $[(M-H) - 162 - 162]^-$, which corresponded to the successive loss of two hexosyl moieties.

The ^{1}H and ^{13}C NMR spectra showed signals of the classical ABC rings of an oleanane-type aglycon with five methyl groups as singlets at δ_H 0.44/δ_C 17.1, δ_H 0.63/δ_C 15.2, δ_H 0.97/δ_C 16.3, δ_H 1.14/δ_C 27.7, δ_H 1.23/δ_C 28.1, an olefinic proton at δ_H 5.42 (*br t*, J = 2.9 Hz)/δ_C 121.3 (H-12) and one oxygen-bearing methine protons at δ_H 3.19 (*dd*, J = 11.7, 4.0 Hz)/δ_C 89.4 (H-3). However, strong differences appear in the D ring: an olefinic proton at δ_H 7.18 (*dd*, J = 6.7, 3.0 Hz) (H-16) which correlated in HMBC with δ_C 32.3 (C-15), 43.5 (C-14) and a high-field shifted signal of a α,β insaturated carboxyl function at 170.0 (C-28). The H-18 at δ_H 3.87 gave unusual HMBC correlations at δ_C 138.8 (C-16) and 143.0 (C-17) for the D ring and HMBC cross-peaks at δ_H 3.87/δ_C 45.1 (C-19) and δ_H 3.87/δ_C 33.3 (C-20), suggested a side chain linked at the C-18 position instead of the E ring. This chain possessed a primary alcoholic function at C-22 deduced by the HMBC correlation at δ_H 1.83, 1.93 (H_2-21)/δ_C 66.6 (C-22). These data allowed the identification of the genin as the rare (3β)-3,22-dihydroxy-17,22-secooleana-12,16-dien-28-oic acid, named steganogenin (Table 1), previously isolated from *Steganotaenia araliacea* (Lavaud et al., 1992) and *Pittosporum angustifolium* (Bäcker et al., 2013).

As for compound **3**, the monosaccharides obtained by acid hydrolysis of **4** were identified as D-glucose (Glc) in a β anomeric orientation in its pyranose form.

The ^{1}H NMR spectrum of **4** showed signals of three anomeric protons at δ_H 4.76 (*d*, J = 7.4 Hz), 4.77 (*d*, J = 7.4 Hz) and 5.27 (*d*, J = 7.4 Hz), which gave correlations in the HSQC spectrum with three anomeric carbon signals at δ_C 103.9, 103.9 and 104.5, respectively. Units of three β-D-glucopyranosyl moieties were identified (Table 2). A HMBC correlation at δ_H 4.76 (*d*, J = 7.4 Hz, Glc1-1)/δ_C 89.4 (C-3) and a NOESY cross-peak at δ_H 4.76 (*d*, J = 7.4 Hz, Glc-1)/δ_H 3.19 (*dd*, J = 11.7, 4.0 Hz, H-3), revealed the linkage with the aglycon. Another correlations in the HMBC spectrum between δ_H 5.27 (*d*, J = 7.4 Hz, Glc2-1) and δ_C 81.5 (Glc1-2), and the reverse one between δ_H 4.17 (Glc1-2) and δ_C 104.5 (Glc2-1), showed the (1 → 2) linkage between Glc2 and Glc1. The linkage of the last β-D-glucopyranosyl moiety Glc3 at the C-22 position of the aglycon was based on the NOESY cross-peak at δ_H 4.77 (*d*, J = 7.4 Hz, Glc3-1)/δ_H 3.77, 4.34 (H_2-22), and the HMBC cross-peak at δ_H 4.77 (*d*, J = 7.4 Hz, Glc3-1)/δ_C 66.6 (C-22).

According to the above results, the structure of **4** was elucidated as the new 3-*O*-β-D-glucopyranosyl-(1 → 2)-glucopyranosyl-22-*O*-β-D-glucopyranosylsteganogenin.

From a chemotaxonomic point of view, the Apiales order contains families as Apiaceae, Araliaceae, and Pittosporaceae, rich in saponins possessing hydroxylated oleanane-type aglycons such as barringtogenol C, A1-barrigenol or R1-barrigenol (Bäcker et al., 2013; Kartal et al., 2006; Manase et al., 2013; Della Greca et al., 1994). More specifically, an unusual structure of aglycon, a 17,22-seco-oleanane skeleton, is found only in the Apiales, in various families. From the Apiaceae family, Saniculoideae sub-family, glycosides of this aglycon were isolated from *S. araliacea* (Lavaud et al., 1992) and *E. kotschyi*, in the Pittosporaceae family, from *P. angustifolium* (Bäcker et al., 2013), and in the Araliaceae family from *Hydrocotyle ranunculoides* (Della Greca et al., 1994). This triterpene might be a chemotaxonomic marker of the Apiales order.

3. Experimental

3.1. General experimental procedures

Optical rotations values were recorded on a AA-OR automatic polarimeter. The 1D and 2D NMR spectra (^{1}H and ^{13}C NMR, ^{1}H–^{1}H COSY, TOCSY, NOESY, HSQC and HMBC) were performed using a UNITY-600 spectrometer at the operating frequency of 600 MHz on a Varian INOVA 600 instrument equipped with a SUN 4 L-X computer system (600 MHz for ^{1}H and 150 MHz for ^{13}C spectra). Conventional pulse sequences were used for COSY, HSQC, and HMBC spectra. TOCSY spectra were acquired using the standard MLEV17 spin-locking sequence and a 90 ms mixing time. The mixing time in the NOESY experiment was set to 500 ms. The carbon type (CH_3, CH_2, CH) was determined by DEPT experiments. Chemical shifts are reported as δ values (ppm), referenced with respect to the residual solvent signal of C_5D_5N, and coupling constants (J) were measured in Hz. The samples were solubilised in pyridine-d_5. HRESIMS (positive-ion mode) was carried out on a Q-TOF 1-micromass spectrometer. FABMS were conducted in the negative-ion mode on a Jeol SX-102 instrument. Isolation of compounds were carried out using column chromatography (CC) on Silica gel 60 (Merck, 63–200 μm), medium-pressure liquid chromatography (MPLC) on silica gel 60 (Merck, 15–40 μm) with a Master Flex L/S Digital Economy Drive MPLC pump equipped with Spectra/Chrome LC column (110 × 250 mm) connected with Foxy 200 Fraction collector and a Gilson M 305 pump (25 SC head pump, M 805 manometric module), a Büchi glass column (460 mm × 25 mm and 460 mm × 15 mm), and a Büchi precolumn (110 mm × 15 mm), and MPLC on reversed-phase RP-18 silica gel (75–200 μm, Silicycle) with a Gilson Pump Manager C-605, having two pumps (2 × Büchi pump module C-601). Thin-layer chromatography (TLC, Silicycle) and high-performance thin-layer chromatography (HPTLC, Merck) were carried out on precoated silica gel plates $60F_{254}$. The spray reagent for saponins was Komarowsky reagent (2% 4-hydroxybenzaldehyde in MeOH – 50% H_2SO_4, 5:1).

3.2. Plant material

The roots of *E. kotschyi* Boiss. were collected in August 2005 from Hadim-Konya, Turkey and identified by Prof. Dr. Hayri Duman (Department of Biology, Faculty of Sciences, Gazi University, Ankara). A voucher specimen (AEF 25381) was deposited in the Herbarium of the Faculty of Pharmacy, Ankara University, Ankara, Turkey.

3.3. Extraction and isolation

The dried powdered roots of *E. kotschyi* (400 g) were extracted with methanol using soxhlet apparatus (17 h). After evaporation of the solvent, 90.7 g of the residue was then dissolved in 300 mL of H_2O and successively partitioned with hexane (1100 mL), dichloromethane (955 mL), and H_2O-saturated *n*-BuOH, respectively, yielding after evaporation of the solvents hexane (2.4 g), dichloromethane (1.3 g), H_2O-saturated *n*-BuOH (46.2 g) and the remaining H_2O (30.3 g) extracts.

The *n*-BuOH extract was submitted to column chromatography on silica gel, eluted with a gradient system (CH_2Cl_2–MeOH–H_2O 80/20/2, 70/30/3, 50/50/5, MeOH) yielding 7 main fractions (Fr.1–7). Fr.5, rich in saponins, was submitted to a MPLC on reverse-phase silica gel RP-18, eluted with a MeOH–H_2O gradient (30/70 to 100/0) to afford 15 subfractions. Further separations of these subfractions were performed by successive MPLC on silica gel ($CHCl_3$–MeOH–H_2O 31/17/2, 13/7/2) and purification was achieved on reversed-phase silica gel RP-18 (MeOH–H_2O gradient) to give compounds **1** (16.2 mg), **2** (5.5 mg), **3** (11.4 mg), **4** (14.0 mg), and 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucuronopyranosyloleanolic acid (3.4 mg) (Melek et al., 2003). Fr.7, rich in saponins too, was applied to MPLC on silica gel (gradient $CHCl_3$–MeOH–H_2O 40/10/1 to 60/30/7) to give 10 subfractions, then purified by MPLC on reversed-phase silica gel RP-18 (MeOH–H_2O gradient) yielding 3-*O*-β-D-galactopyranosyl-(1 → 2)-[α-L-arabinopyranosyl-(1 → 3)]-β-D-glucuronopyranosyl-22-*O*-angeloylA1-barrigenol (26.5 mg) (Bäcker et al., 2013).

3.4. Compound characterization

3.4.1. 3-O-α-L-Rhamnopyranosyl-(1 → 4)-β-D-glucuronopyranosyl-22-O-β,β-dimethylacryloylA1-barrigenol (1)

White, amorphous powder; $[\alpha]_D^{25} = -25$ (c 0.05, MeOH); ^{1}H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz), see Tables 1 and 2; FABMS (negative-ion mode) m/z 893 [M−H]$^-$, 747 [(M−H) − 146]$^-$, HRESIMS (positive-ion mode) m/z 918.4880 [M+Na]$^+$ (calcd. for $C_{47}H_{74}O_{16}Na$, 918.4875).

3.4.2. 3-O-α-L-Rhamnopyranosyl-(1 → 4)-β-D-glucuronopyranosyl-22-O-angeloylA1-barrigenol (2)

White, amorphous powder; $[\alpha]_D^{25} = -18$ (c 0.05, MeOH); ^{1}H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz), see Tables 1 and 2; FABMS (negative-ion mode) m/z 893 [M−H]$^-$, 747 [(M−H) − 146]$^-$, HRESIMS (positive-ion mode) m/z 918.4880 [M+Na]$^+$ (calcd. for $C_{47}H_{74}O_{16}Na$, 918.4875).

3.4.3. 3-O-β-D-Glucopyranosyl-(1 → 2)-[β-D-glucopyranosyl-(1 → 6)]-β-D-glucopyranosyl-21,22,28-O-triacetyl-(3β,21β,22α)-olean-12-en-16-one (3)

White, amorphous powder; $[\alpha]_D^{25} = +8$ (c 0.10, MeOH); ^{1}H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz), see Tables 1 and 2; FABMS (negative-ion mode) m/z 1099 [(M−H)]$^-$, 1056 [(M−H) − 43]$^-$, 894 [(M−H) − 43 − 162]$^-$, HRESIMS (positive-ion mode) m/z 1123.5312 [M+Na]$^+$ (calcd. for $C_{54}H_{84}O_{23}Na$, 1123.5301).

3.4.4. 3-O-β-D-Glucopyranosyl-(1 → 2)-glucopyranosyl-22-O-β-D-glucopyranosylsteganogenin (4)

White, amorphous powder; $[\alpha]_D^{25} = -16$ (c 0.20, MeOH); ^{1}H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz), see Tables 1 and 2; ESIMS (negative-ion mode) m/z 957 [(M−H)]$^-$, 795 [(M−H) − 162]$^-$, 633 [(M−H) − 162 − 162]$^-$, HRESIMS (positive-ion mode) m/z 981.5039 [M+Na]$^+$ (calcd. for $C_{48}H_{78}O_{19}Na$, 981.5035).

3.5. Acid hydrolysis and GC analysis

Each compound (3 mg) was hydrolyzed with 2 N aq. CF_3COOH (5 mL) for 3 h at 95 °C. After extraction with CH_2Cl_2 (3 × 5 mL), the aq. layer was repeatedly evaporated to dryness with MeOH until neutral, and then analyzed by TLC over silica gel ($CHCl_3$–$MeOH$–H_2O 8/5/1) by comparison with authentic samples. Furthermore, the residue of sugars was dissolved in anhydrous pyridine (100 μL), and L-cysteine methyl ester hydrochloride (0.06 mol/l) was added. The mixture was stirred at 60 °C for 1 h, then 150 μL of HMDS–TMCS (hexamethyldisilazane–trimethylchlorosilane 3/1) was added, and the mixture was stirred at 60 °C for another 30 min. The precipitate was centrifuged off, and the supernatant was concentrated under a N_2 stream. The residue was partitioned between *n*-hexane and H_2O (0.1 mL each), and the hexane layer (1 μL) was analyzed by GC (Hara et al., 1987). The absolute configurations were determined by comparing the retention times with thiazolidine derivatives prepared in a similar way from standard sugars (Sigma–Aldrich).

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